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 TI **Cis-Hydroxyproline** inhibits proliferation, collagen synthesis, attachment, and migration of culture bovine retinal pigment epithelial cells.
 AU Yoo, Jin-Seong; Sakamoto, Taiji; Spee, Christine; Kumura, Hideya; Harris, Michael S.; Hinton, David R.; Kay, Eunduck P.; Ryan, Stephen J. (1)
 CS (1) Doheny Eye Inst., Univ. Southern California, 1450 San Pablo St., Los Angeles, CA 90033 USA
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 AB Purpose. Proliferative vitreoretinopathy (PVR) is characterized by the proliferation and migration of retinal pigment epithelial (RPE) and other cell, into the vitreous cavity. The PVR membrane formation also is associated with collagen production by RPE. The authors examined the effect of a proline analog, **cis-hydroxyproline** (CHP), on proliferation, collagen synthesis, attachment, and migration of bovine RPE in vitro. Methods. The effect of CHP on cell proliferation was determined as a function of dosage and days in culture by counting the cell numbers on days 3, 6, and 9. Collagen synthesis was determined by trichloroacetic acid precipitation of the radiolabeled samples before and after bacterial collagenase digestion. The attachment **assay** involved type I collagen or fibronectin substrates or both (2.5 mu-g/well). For migration experiments, RPE cells were removed from a defined area of a confluent culture, and migration was quantitated by counting the number of cells migrating into the denuded area over 30 hours. Results. The addition of CHP inhibited RPE proliferation in both a dose- and a time-dependent manner, collagen synthesis, attachment, and migration also were inhibited by CHP in a dose-dependent manner. When the culture plates were coated with collagen, 1t 100 mu-g/ml of CHP had no effect on cell attachment. Higher doses of CHP resulted in mild inhibition of attachment on collagen-coated plates. Simultaneous addition of L-proline to the cultures resulted in blockade of these inhibitory effects on proliferation, collagen synthesis, attachment, and migration. Conclusions. The results show that RPE functions critical to the development of PVR are inhibited by CHP, suggesting the possibility that this drug may have potential clinical application.

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cis-Hydroxyproline Inhibits Proliferation, Collagen Synthesis, Attachment, and Migration of Cultured Bovine Retinal Pigment Epithelial Cells

Jin-Seong Yoo,†‡ Taiji Sakamoto,*† Christine Spee,*† Hideya Kimura,*† Michael S. Harris,*† David R. Hinton,‡ EunDuck P. Kay,*† and Stephen J. Ryan*†*

Purpose. Proliferative vitreoretinopathy (PVR) is characterized by the proliferation and migration of retinal pigment epithelial (RPE) and other cells into the vitreous cavity. The PVR membrane formation also is associated with collagen production by RPE. The authors examined the effect of a proline analog, *cis*-hydroxyproline (CHP), on proliferation, collagen synthesis, attachment, and migration of bovine RPE in vitro.

Methods. The effect of CHP on cell proliferation was determined as a function of dosage and days in culture by counting the cell numbers on days 3, 6, and 9. Collagen synthesis was determined by trichloroacetic acid precipitation of the radiolabeled samples before and after bacterial collagenase digestion. The attachment assay involved type I collagen or fibronectin substrates or both (2.5 µg/well). For migration experiments, RPE cells were removed from a defined area of a confluent culture, and migration was quantitated by counting the number of cells migrating into the denuded area over 30 hours.

Results. The addition of CHP inhibited RPE proliferation in both a dose- and a time-dependent manner; collagen synthesis, attachment, and migration also were inhibited by CHP in a dose-dependent manner. When the culture plates were coated with collagen, <100 µg/ml of CHP had no effect on cell attachment. Higher doses of CHP resulted in mild inhibition of attachment on collagen-coated plates. Simultaneous addition of *L*-proline to the cultures resulted in blockade of these inhibitory effects on proliferation, collagen synthesis, attachment, and migration.

Conclusions. The results show that RPE functions critical to the development of PVR are inhibited by CHP, suggesting the possibility that this drug may have potential clinical application. *Invest Ophthalmol Vis Sci.* 1997;38:520–528.

Recent advances in vitreoretinal surgery have improved the prognosis of rhegmatogenous retinal detachment,^{1–3} but long-term success often is impeded by the development of proliferative vitreoretinopathy (PVR), a disease in which cellular membranes form

within the vitreous cavity and along the retinal surfaces. Membrane formation and subsequent contraction can have a devastating effect on vision, and this process is responsible for the majority of failures of retinal reattachment surgery.^{4–6} A variety of drugs, ranging from antimetabolites to steroids, have been tested as potential therapeutic agents for the treatment of PVR; however, many of the more effective inhibitors of cellular proliferation also have toxic side effects on the eye.^{7–14} Thus far, only one of these agents, fluorouracil, has been tested in patients.¹⁵

In a continuing search for a safe and effective antiproliferative drug for intraocular use, we recently have evaluated a proline analog, *cis*-hydroxyproline (CHP). Proline analogs are incorporated into polypeptides, replacing the proline residues and altering

From the *Doheny Eye Institute and the Departments of †Ophthalmology and ‡Pathology, University of Southern California School of Medicine, Los Angeles, California; and the Department of §Ophthalmology, Catholic University Medical College, Seoul, Korea.

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Reprint requests: Stephen J. Ryan, Doheny Eye Institute, University of Southern California, 1450 San Pablo Street, Los Angeles, CA 90033.

the structure and function of the corresponding polypeptides. These analogs inhibit the production of pro-collagen on the post-translational level by preventing the polypeptides from folding into a stable triple-helical conformation.¹⁶⁻¹⁸ The nonhelical polypeptides then are readily susceptible to proteolysis by intracellular enzymes,^{7,16,19} leading to reduced secretion and reduced deposition of extracellular collagen fibers. In fibroblasts, the rate of translation of collagenous polypeptides and the pro-collagen messenger RNA activity is not affected by the analogs.²⁰ Proline analogs inhibit the adherence and proliferation of cultured fibroblasts^{20,21} and the migration of epidermal and cultured endothelial cells.^{22,23} Published reports suggest that collagen production may be important in many aspects of cell behavior. Retinal pigmented epithelial (RPE) cells are capable of producing interstitial collagen and may play a role in wound healing and in diseases such as PVR.^{24,25} In this study, we report the inhibitory effects of a proline analog, *cis*-hydroxyproline (CHP), on the proliferation, collagen synthesis, attachment, and migration of bovine RPE cells in culture.

MATERIALS AND METHODS

Cell Culture

Bovine eyes were obtained from a local abattoir and the RPE cells collected by a previously described method.²⁶ Briefly, the eye was bisected and the anterior portion was removed, leaving the intact optic cup. After resection of the retina, medium containing 0.1% trypsin and 0.02% ethylenediaminetetraacetic acid (Sigma, St. Louis, MO) was instilled into the eye cup and incubated for 10 minutes at 37°C. The medium and the dissociated cells therein were collected by gentle pipetting, seeded in six-well culture plates previously coated with laminin (Collaborative Research, Bedford, MA), and incubated with Dulbecco's modified Eagle's medium (DMEM; Irvine Scientific, Santa Ana, CA) containing 10% fetal bovine serum (FBS, Irvine Scientific), 100 µg/ml streptomycin, and 100 µg/ml penicillin (DMEM-10). The cells were confirmed to be RPE cells by their typical morphology and by their keratin positivity as shown by immunocytochemistry.^{27,28} All experiments conformed to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research.

Proliferation Assay

The RPE cells (1×10^4 /well) were seeded on a 24-well plate. Cells were incubated in DMEM-10 for 24 hours and allowed to attach to the well. Then the cells were incubated in DMEM-10 in the presence or absence of CHP (1, 10, 50, 100 µg/ml, Sigma), with

or without the addition of *L*-proline (200 µg/ml). The medium was changed every third day. After incubation for 3, 6, or 9 days, the number of cells in each well was counted with a hemocytometer. Cell viability was assessed by trypan blue exclusion.²⁹ All experiments were done in triplicate and repeated three times.

³H-Thymidine Incorporation to Assess DNA Synthesis

To determine the effect of CHP on DNA synthesis of RPE cells, a ³H-thymidine uptake assay was performed in the presence or absence of CHP (0.1, 1.0, 10.0, 50.0, 100.0 µg/ml), with or without *L*-proline (200 µg/ml). The RPE cells (1.0×10^4 cells/well) were seeded on a 24-well culture plate and incubated with DMEM-10 for 24 hours. Next, RPE cells were incubated in medium containing various concentrations of CHP with or without *L*-proline. After 24 hours, 0.5 µCi ³H-thymidine (Amersham, Arlington Heights, IL) was added to each well. Cells then were further incubated for 20 hours, after which the medium was removed. Next, 7% trichloroacetic acid (TCA) was added for 30 minutes at 4°C, then removed and discarded. Finally, 0.2-M sodium hydroxide was added for 20 minutes at room temperature, then collected in scintillation tubes, added to 10-ml scintillation fluid (ICN Chemicals, Costa Mesa, CA), and counted in a Beckman scintillation counter. Each experiment was done in quadruplicate and repeated three times.

Collagen Synthesis

Collagen synthesis was determined using a modified version of the method described by Diegelmann et al and Low et al.^{30,31} The RPE cells that were trypsinized and suspended in DMEM-10 were seeded into the wells (3×10^4 cells/well) of 24-well culture plates. Five days after plating, confluent cells were treated for the first 24 hours with 1-ml DMEM-10 containing appropriate concentrations of CHP (1, 10, 50, 75, 100 µg/ml), with or without *L*-proline (200 µg/ml). After exposure to CHP, the cells were pulsed with 1-ml DMEM containing 2% FBS, 40 µCi/ml ³H-proline (43 Ci/mmol; Amersham, Arlington Heights, IL), 50 µg/ml ascorbic acid, and various concentrations of CHP. The cells were incubated for 24 hours in 5% carbon dioxide at 37°C. The pulse was terminated by heating the plate for 10 minutes at 90°C and sonicating for 30 seconds per well at 40% power (Biosonik III, Rochester, NY). One fifth of a milliliter of the cell homogenate was removed from each well for protein determination using a dye-binding assay.³² Total protein was precipitated with approximately 5% TCA, and washed five times by repeated centrifugation at 2000 rpm (Beckman Model J6-B, Palo Alto, CA), aspiration of the supernatant, and precipitation with 10% TCA in a 1-mM solution of cold *L*-proline (Sigma). Trichloro-

acetic acid was extracted by addition of cold ethanol:ethyl ether (3:1) after the last centrifugation, and the dried protein was incubated in 0.5 ml of an incubation buffer containing 60- μ mol Hepes buffer (pH 7.2), 1.25 μ mol N-ethylmaleimide, 0.25 μ mol CaCl₂ for 4 hours in a shaking water bath (80 cycles/min) at 37°C. Next, the protein suspensions were precipitated with 0.5 ml of a 5% TCA solution followed by 0.5 ml of a 10% TCA solution, and the supernatants were saved as the incubation blank. Total protein was again freed of TCA and dried. This was digested with 10 vial units of bacterial collagenase form III (Advanced Biofactors, Lynbrook, NY) in 0.5-ml incubation buffer under the same conditions as described for the incubation blank. Using this collagenase, an incubation time of 4 hours was shown to be optimal for complete collagen digestion.³⁴ Noncollagen protein (NCP) was precipitated in 5% and 10% TCA solutions, and the supernatants were saved as the collagenase-digestible protein (CDP). The pellets were dissolved with 0.5 ml of 0.2 N sodium hydroxide followed by 1 ml of 0.2 N hydrochloric acid and saved as the noncollagen protein fraction. All manipulations after sonication were performed at 4°C. Aliquots of each incubation blank, collagen fraction, and noncollagen fraction were placed in scintillation vials with Ready Protein scintillation cocktail (ICN Chemical, Costa Mesa, CA) in a 1:10 dilution and vortexed. Radioactivity in each vial was counted for 1 minute using a Beckman scintillation counter. For each well, percent collagen synthesized relative to total protein was calculated by an equation similar to that used to assay chick embryo collagen synthesis.³⁵

%Collagen

$$= \frac{\text{cpm in CDP}}{(\text{cpm in NCP} \times 4.4) + (\text{cpm in CDP})} \times 100$$

The RPE cells produce a wide spectrum of collagen types, including fibrillar collagens (types I, III, V) and basement membrane collagens (type IV)^{24,25}; however, the proportions of collagen types produced are not known specifically. The percent collagen present in a portion of analyzed protein substrate was determined on the basis of the following calculations: the sum of proline plus hydroxyproline in bovine collagen is approximately 22.4% of the amino acid residues, whereas an average noncollagen protein contains approximately 5.1% proline and hydroxyproline.³⁶ Therefore, bovine collagen contains approximately 4.4 times more proline and hydroxyproline than does noncollagen protein.

Attachment Assay

Twenty-four well tissue culture plates were coated with human fibronectin or rat-tail type I collagen (Collabo-

rative Research, Bedford, MA). The proteins were dissolved in Dulbecco's phosphate buffered saline at 5 mg/l, and each well was incubated with 0.5 ml of this solution for 5 hours at room temperature. To determine the effect of both collagen and fibronectin, the plate was incubated with 0.5 ml per well of each solution. For attachment assays, confluent, third through fifth passage RPE cells were inoculated into 75-cm² flasks in DMEM-10 with CHP (0 to 400 μ g/ml) or L-proline (200 μ g/ml) or both for 24 hours. Cells were trypsinized and suspended in medium supplemented with 2% FBS and the analog used at the same concentrations as in the preincubation. The cells (1 \times 10⁴/well) were seeded on the 24-well tissue culture plates and incubated for 50 minutes; the wells were rinsed twice with Hank's balanced salt solution, and both rinses were combined. Trypsin (0.1%) was added to minimize cell clumping before these unattached cells were counted using an electronic cell counter. The cells that remained attached to the wells were trypsinized and counted also.

Migration Assay

The migration assay was performed by our previously described method with only minor modifications. The RPE cells were seeded into 20-mm well diameter 12-well plates (Corning Glass Works, Corning, NY) at a density of 3 \times 10⁴ cells per well. The cells were grown to confluence, and 2 days later, the assay was performed. A straight-edge razor blade was used to wound the monolayer and denude two areas (10 \times 5 mm) in each well of cells. Twenty-four hours before wounding, cultures were treated with various concentrations of CHP (0, 1, 10, 50, 100 μ g/ml), with or without L-proline (200 μ g/ml), in DMEM-10. Migration was evaluated by quantitating migration of cells into a denuded area of the cultures. The cells were washed four times with Hank's balanced salt solution to remove floating cells, and the analog was added, at the same concentrations as in the preincubation, in medium supplemented with 2% FBS. After 30 hours, the cells were fixed with 8% paraformaldehyde solution at 4°C for 5 minutes and stained with Richardson's stain at room temperature for 10 minutes. Migration was quantified by counting the number of cells that migrated into the denuded area per microscopic field. The wound edge was identified by the light scratch left by the razor blade on the surface of the well.

Statistical Analysis

All experimental data were analyzed statistically using the paired two-tailed Student's *t*-test between experimental group and control.

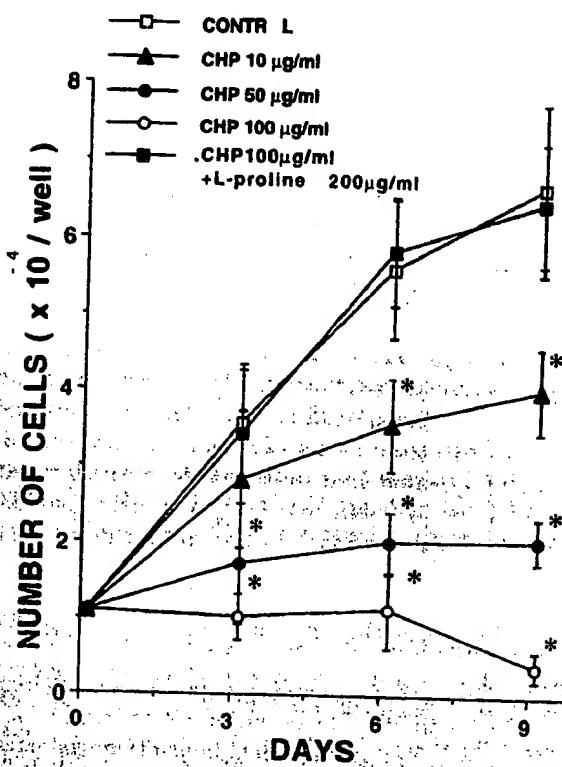


FIGURE 1. Dose-dependent and time-dependent inhibition of retinal pigment epithelial proliferation by varying concentrations of *cis*-hydroxyproline. Cells, plated on plastic culture plates, were allowed to attach for 24 hours before the addition of the analog in the concentration indicated. This inhibitory effect was abolished by the addition of *L*-proline (200 µg/ml) to the medium. All data are expressed as a mean \pm standard deviation ($n = 9$). *Significant difference ($P < 0.01$) from control proliferation of each interval at each concentration.

RESULTS

Cell Proliferation Assay

cis-Hydroxyproline inhibited the proliferation of bovine RPE cells in a dose-dependent and a time-dependent manner. At 6 and 9 days, CHP concentrations of 10 µg/ml or more significantly reduced the number of cells, but at 3 days, only CHP concentrations of 50 and 100 µg/ml significantly reduced cell numbers (Fig. 1). Inhibition dose 50 (ID₅₀) values were 47.5, 30.0, and 23.0 µg/ml at 3, 6, and 9 days, respectively. In the ³H-thymidine uptake assay, thymidine uptake by RPE cells also decreased in a dose-dependent manner, with an ID₅₀ value of 60 µg/ml (Fig. 2). The inhibitory effect was abolished by adding *L*-proline (200 µg/ml) to the medium. These findings were consistent with those of a preliminary study that had shown *L*-proline itself had no effect on cell proliferation (data not shown). Cells cultured with the CHP became flattened and rounded, whereas cells in the control culture and

in the culture with both the analog and *L*-proline retained both spindle and polygonal morphologies typical of RPE (Fig. 3).

Collagen Synthesis

Treatment with CHP inhibited the newly synthesized CDP in the culture of RPE cells in a dose-dependent manner; at 50, 75, and 100 µg/ml, CHP inhibited CDP 40%, 70%, and 90%, respectively (Table 1). At a concentration of 100 µg/ml, CHP inhibited NCP 10% by assay with bacterial collagenase (Table 1), which digests CDP into TCA-soluble peptides but leaves NCP undigested and thus TCA precipitable. The CHP at 50, 75, and 100 µg/ml inhibited collagen synthesis by 35%, 65%, and 87%, respectively. Simultaneous treatment with proline prevented the inhibitory effect of CHP on collagen synthesis (Table 1).

Attachment Assay

Control RPE cultures showed varying degrees of attachment depending on the substrate. Within 5 minutes, 31%, 50%, and 54% of RPE cells attached to wells coated with type I collagen, fibronectin and both fibronectin and type I collagen, respectively. In contrast, during the same periods, only 15% of the cells attached to plastic substrate with or without *L*-proline (Fig. 4).

The RPE attachment to defined substrates was inhibited by varying concentrations of CHP (Fig. 5). *cis*-Hydroxyproline inhibited the number of cells that attached to the plastic substratum of culture plates in a dose-dependent manner (Fig. 5, open squares). Its

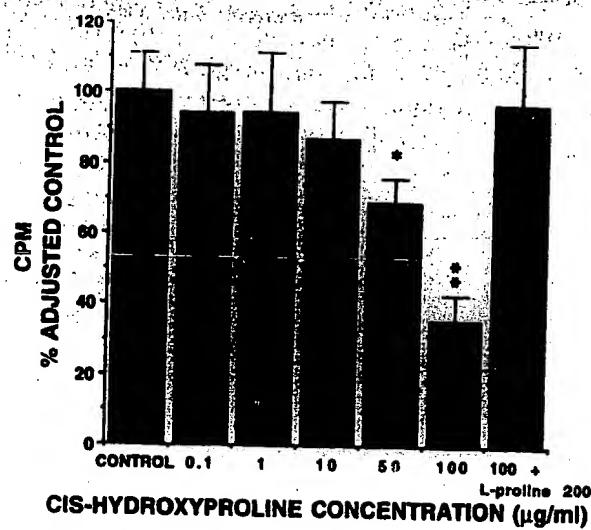


FIGURE 2. Dose response curve of the inhibitory effect of *cis*-hydroxyproline on ³H-thymidine uptake. Data are expressed as a mean \pm standard deviation ($n = 12$). Asterisks indicate a significant difference from the control of each concentration (* $P < 0.05$, ** $P < 0.01$).

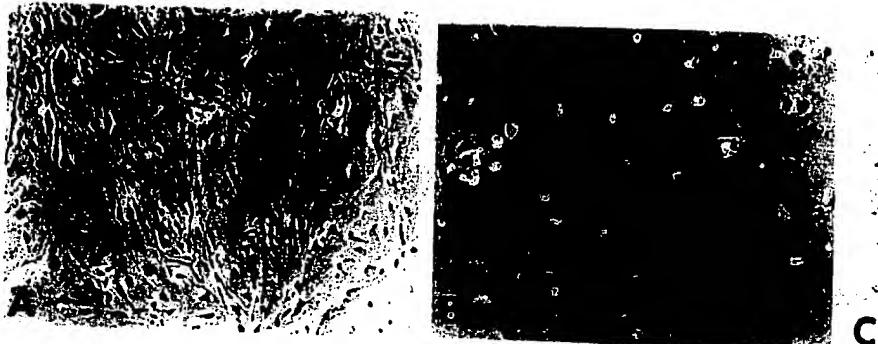


FIGURE 5. Photomicrographs of *cis*-hydroxyproline (CHP)-treated retinal pigment epithelial cells after 3 days. (A) Control (original magnification, $\times 56$); (B) $100 \mu\text{g}/\text{ml}$ of CHP (original magnification, $\times 90$); (C) $100 \mu\text{g}/\text{ml}$ of CHP plus $200 \mu\text{g}/\text{ml}$ of *L*-proline (original magnification, $\times 56$). The cells in the control culture and culture with CHP and *L*-proline exhibited typical spindle or polygonal shapes, whereas the cells in the culture with CHP had a flattened, rounded shape. The cells in the control and CHP plus *L*-proline culture had reached confluence, whereas cell proliferation in the CHP at $100 \mu\text{g}/\text{ml}$ culture was inhibited.

inhibitory effect was attenuated by adding *L*-proline to the medium (Fig. 5, open circles). *L*-proline ($200 \mu\text{g}/\text{ml}$) alone had no effect on cell attachment (data not shown). Prior coating of culture plates with collagen or fibronectin reduced the inhibitory effect of CHP on cell attachment. Culture plates coated with collagen did not show the inhibitory effect of low doses of CHP ($<100 \mu\text{g}/\text{ml}$) on cell attachment, whereas at high doses of CHP ($>200 \mu\text{g}/\text{ml}$), the collagen-coated plates exhibited only a modest inhibition of RPE attachment (Fig. 5, filled triangles). Culture plates coated with fibronectin showed partial inhibition of attachment by CHP; at 100 , 200 , and $400 \mu\text{g}/\text{ml}$, CHP inhibited the attachment 18% , 25% and 30% , respectively (Fig. 5, filled squares). The culture plates coated previously with both collagen and fibro-

nectin completely blocked the effect of CHP on attachment, even at high doses of CHP (Fig. 5, filled circles).

Cell Migration Assay

cis-Hydroxyproline inhibited the migration of RPE cells into the denuded area in a dose-dependent manner. Addition of CHP at $50 \mu\text{g}/\text{ml}$ inhibited migration by 38% , whereas CHP at $100 \mu\text{g}/\text{ml}$ inhibited migration by 72% . Simultaneous treatment with *L*-proline ($200 \mu\text{g}/\text{ml}$) blocked the inhibitory effect of CHP on migration (Figs. 6, 7).

DISCUSSION

In certain pathologic conditions, RPE cells have been shown to produce excessive amounts of extracellular

TABLE 1. Effects of CHP on Protein Synthesis of Collagenous and Noncollagenous Protein by RPE Cell

CHP ($\mu\text{g}/\text{ml}$)	CDP ($\text{cpm}/\mu\text{g}$ protein)	NCP ($\text{cpm}/\mu\text{g}$ protein)	Collagen Synthesis (%)
0 (control)	384 ± 12	2730 ± 13	3.1 ± 0.2
1	393 ± 13	2645 ± 70	3.3 ± 0.1
10	360 ± 10	2613 ± 94	3.0 ± 0.2
50	$235 \pm 17^*$	2634 ± 64	$2.0 \pm 0.1^*$
75	$117 \pm 11^*$	2558 ± 61	$1.1 \pm 0.2^*$
100	$40 \pm 60^*$	$2434 \pm 89^{\dagger}$	$0.4 \pm 0.1^*$
<i>L</i> -proline (200)	424 ± 58	2829 ± 146	3.3 ± 0.4

Results are mean \pm SD ($n = 5$).

CHP = *cis*-hydroxyproline; RPE = retinal pigment epithelial; CDP = collagenase-digestible protein; NCP = noncollagenous protein.

* $P < 0.001$ versus control; $\dagger P < 0.05$ versus control; $\ddagger P < 0.01$ versus control.

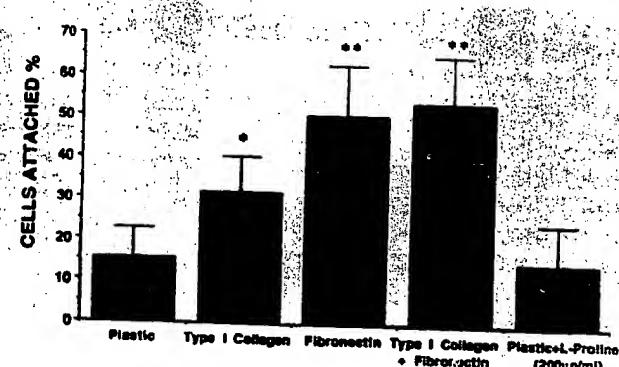


FIGURE 4. Attachment assay using culture plates coated previously with type I collagen or fibronectin or both. Control retinal pigment epithelial cultures showed varying degrees of attachment depending on the substrate. Results are given as a mean \pm standard deviation ($n = 9$). The asterisks indicate difference from the plastic substratum (* $P < 0.05$, ** $P < 0.01$). *L*-proline ($200 \mu\text{g}/\text{ml}$) added to the medium had no effect on cell attachment.

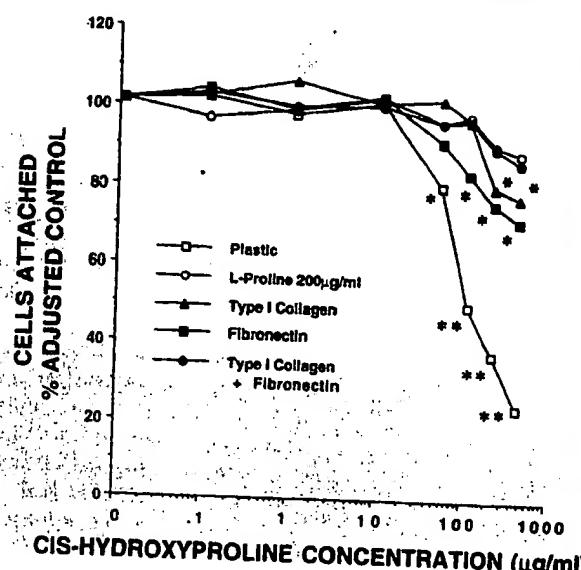
Effect of *cis*-Hydroxyproline on Retinal Pigment Epithelium

FIGURE 5. Inhibition of retinal pigment epithelial attachment to defined substrates by varying concentrations of *cis*-hydroxyproline. Cell attachment is normalized to control values (100%) for each substrate. Each point is a mean \pm standard deviation ($n = 9$). The asterisks indicate a significant difference from the control culture (* $P < 0.05$, ** $P < 0.01$).

matrix material.^{37,38} The production of collagen and glycoproteins by RPE cells may be important in the pathogenesis of PVR. Administration of low doses of *cis*-hydroxyproline (CHP) has been shown previously to reduce collagen deposition in the tissue of several

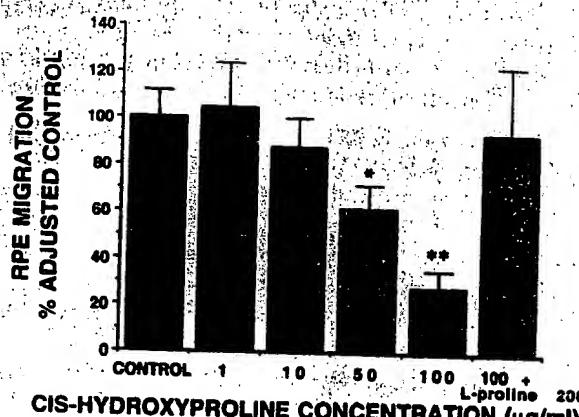


FIGURE 6. The inhibitory effect of varying concentrations of *cis*-hydroxyproline on retinal pigment epithelial cell migration. The migration of retinal pigment epithelial cells was measured as the number of cells that crossed the wound edge. Results are given as mean \pm standard deviation ($n = 6$). The inhibitory effect was reversed by adding *L*-proline to the medium. The asterisks indicate a significant difference from the control for each concentration (* $P < 0.05$, ** $P < 0.01$).

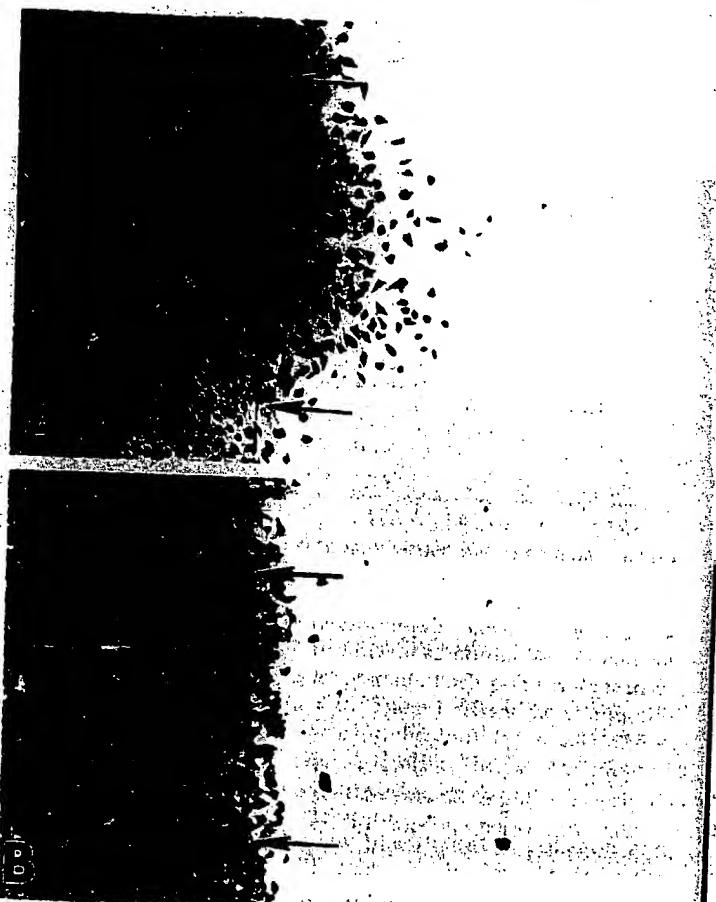


FIGURE 7. Photomicrographs of retinal pigment epithelial migration (Dulbecco's modified Eagle's medium with 2% fetal bovine serum). Arrows indicate the original wound edge. Cells were fixed and stained with Richardson's stain 30 hours after wounding and treatment. (A) Medium only (control, original magnification, $\times 29$); (B) 100 μ g/ml of *cis*-hydroxyproline (original magnification, $\times 28$).

animal models without producing toxicity.³⁹ Studies on aorta-derived cells *in vitro* showed that incorporation of CHP into protein did not have any effect on the synthesis and secretion of elastin, whereas no intact procollagen molecules were recovered.^{40,41} Thus, CHP can have a specific effect on collagen synthesis and secretion *in vivo* and *in vitro*.

We showed that CHP, a proline analog, inhibited proliferation, collagen synthesis, attachment, and migration of cultured bovine RPE cells and that these effects were abolished by adding *L*-proline to the medium. The cells cultured with CHP exhibited a flattened, rounded appearance, whereas the cells in the control culture and in the culture with both the analog and *L*-proline retained a spindle and polygonal shape, suggesting that cellular attachment, motile activity, or the cytoskeleton had been altered by this analog. These findings indicate that the continuous produc-

tion of collagen may be required for the attachment and proliferation of RPE cells,⁴² although it is possible that the structure of some noncollagenous protein such as fibronectin, which also is essential for cellular activity, may have been altered in the presence of the analog.

In our experiments, the effective dose of CHP is approximately 50 $\mu\text{g}/\text{ml}$. This dose is higher than that required for skin fibroblasts²¹ but is lower than that required for tumor cells.⁴³ Fibroblasts accumulate greater amounts of collagen than do RPE, whereas previously studied tumor cells appear to accumulate less. These results suggest that the amount of accumulated collagen may correlate directly with the sensitivity of the cells to CHP. The effect of CHP on cell proliferation appeared to be dependent on the incubation period. Low concentrations of CHP (10 $\mu\text{g}/\text{ml}$) did not inhibit thymidine uptake or cell number at 48 hours but reduced cell number only after 6 or 9 days of incubation. Because the incorporation of ¹⁴C-proline is inhibited by CHP depending on the length of the incubation period,⁴⁴ our results support the idea that proline analogs are incorporated into polypeptides in a time-dependent manner.

In our study, lower doses of CHP (<100 $\mu\text{g}/\text{ml}$) inhibited the attachment of cells on the plastic substratum but had no effect on their ability to attach to collagen-coated plates. This conclusion is supported by the finding that addition of exogenous collagen matrix to the culture plate can reverse the effects of CHP on RPE cells and supports the notion that collagen plays an important role in RPE cell attachment. When high doses of CHP (200 and 400 $\mu\text{g}/\text{ml}$) were used, coating the culture plate with a collagen matrix only partially reversed the effect of the CHP. However, this effect was stronger than that produced by fibronectin coating alone. Even at the highest doses tested, CHP did not affect cell attachment to culture plates coated previously with both fibronectin and collagen. These data suggest that although CHP had pronounced effects on CDP, it also had a significant effect on NCP at concentrations of 100 $\mu\text{g}/\text{ml}$ and above. Thus, it is possible that at high doses of CHP, the presence of defective collagen containing CHP within the cells could have an effect on the metabolism of other proteins that participate in cell growth such as fibronectin. Similarly, high doses of CHP inhibit tumor cell proliferation, even though the tumor cells may not require a collagen substrate for growth.⁴³ This conclusion is supported by the finding that CHP (200 $\mu\text{g}/\text{ml}$) decreased the secretion of laminin and heparan sulfate proteoglycan, as well as collagen types I, III, and IV in Schwann cell cultures.⁴⁵ Proline analogs are incorporated randomly into all proline-containing proteins, replacing the same proportion of proline residues in CDP and NCPs. Although we did not deter-

mine whether CHP-containing NCPs retained biological activity, the known influence of proline residues on the secondary structure of polypeptide chains⁴⁶ suggests that proline analogs might alter the conformation and thus the activity of important NCPs.

Cell migration also was inhibited by CHP. Simultaneous treatment with L-proline blocked the inhibitory effect of CHP on migration, suggesting the continuous production of collagen may be required for RPE cell movement. It was not determined whether a reduction in cell attachment in the presence of this analog decreased the migratory activity or whether attachment and migration were attenuated independently by the analog.

In conclusion, we have shown that CHP has dose-dependent inhibition effects on proliferation, collagen production, attachment, and migration of RPE cells in vitro. The results suggest that collagen production plays important direct or indirect roles in these processes and that this proline analog may have potential clinical use to inhibit these RPE functions that are critical to the development of PVR. It will be important to determine the safety of this drug in the eye and its effects on in vivo PVR models.

Key Words:

attachment, α -hydroxyproline, collagen, synthesis, migration, proliferative vitreoretinopathy

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